Appendix A

Mammalian Cell Technology

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SV40 is monkey (Tooze, 1980). Polyoma infection is less efficient than that of SV40, and polyoma vectors are infrequently used. The early gene region and origin of replication of polyoma have been used in vectors to replicate and express foreign genes in mouse cells. The enhancer is unusual in that it consists of two distinct nonoverlapping sequences that have a slightly different tissue specificity (Herbomel et al., 1984).

Deans et al. (1984) have constructed a recombinant vector using the polyoma early-region expression sequences and origin of replication to express immunoglobulin genes in mouse lymphoid cells. They reported very high frequencies of T antigen-producing cells in transient experiments: 35–40% of cells expressed following calcium-phosphate precipitation of the DNA, 60–70% using DEAE-dextran, and 70–80% using protoplast fusion.

2.8.7 Adenovirus and Adeno-Associated Virus

Adenovirus is a human virus with a genome of double-stranded linear DNA, approximately 35 kilobase pairs in length, that encodes at least 30 proteins. Details of its expression and infectious cycle are known (see Tooze, 1980). Adenovirus offers several advantages as a mammalian expression vector (reviewed by Rigby, 1982). Since adenovirus infection inhibits host-cell protein synthesis, genes expressed by adenovirus can account for a greater percentage of total protein synthesis in the cell. A large amount of DNA (up to 7,000 base pairs) can be inserted into the genome without inhibiting viral function (Berkner and Sharp, 1982). Also, the adenovirus major late promoter is a very strong transcriptional promoter. This promoter does not possess an enhancer element, and transcription from this promoter can be further increased by the addition of an enhancer (Kaufman and Sharp, 1982). Adenovirus type 2 is able to replicate in human, simian, and rodent cells.

Among the disadvantages is that adenovirus infection kills the host cell, so that expressing cell lines cannot be established. Also, when adenovirus DNA integrates into the chromosomes of host cells that are nonpermissive for adenovirus replication, only some pieces of the genome integrate, while others are lost (Weinberg, 1980).

The adenovirus major late promoter has been used separately in a number of expression vectors. For instance, it has been used to produce 20- to 40-fold more SV40 large T antigen in monkey cells than is produced by SV40 infection (Thummel et al., 1981); it has been used to express recombinant DHFR gene (Kaufman and Sharp, 1982) and also human erythropoietin (Jacobs et al., 1985).

Adenovirus expresses small transcripts known as VA RNA. This RNA appears to stimulate translation of mRNA species that possess an adenovirus leader sequence. The VA gene has been used in expression vectors to increase the translation efficiency not only of transcripts from the adenovirus major late promoter but also from the SV40 early promoter (Kaufman, 1985).

There is one report of the use of a human parvovirus, adeno-associated virus (AAV), as a mammalian expression vector. Tratschin et al. (1984) transfected the defective AAV genome along with helper adenovirus particles to allow AAV replication and produce infectious AAV particles at high efficiency. A vector was constructed with transcription of the bacterial CAT gene driven by an AAV promoter. After transfection into 293 cells (which express an adenovirus early function) high expression of the CAT gene was obtained without helper virus. In this case, replication did not take place. One advantage of AAV is that it may be possible to control whether the genome integrates into chromosomes or is replicated episomally, depending on whether helper virus is absent or present.

2.8.8 Bovine Papilloma Virus

Bovine papilloma virus (BPV) is a small DNA virus that contains only DNA and protein. Its genome is a double-stranded DNA circle of 7,945 base pairs that has been completely sequenced (Chen et al., 1982). No papilloma virus has yet been cultured in a continuous cell culture system; the complete BPV genome has been cloned into a bacterial plasmid and can therefore be easily propagated and dissected (Danos et al., 1980; Heilman et al., 1980). In fact, BPV cloned into a bacterial plasmid can replicate either in bacteria or in mammalian cells (DiMaio et al., 1982). BPV DNA, either in its entirety or as a subgenomic fragment containing 69% of the original viral DNA, causes morphological transformation of mouse cells in cell culture (Lowy et al., 1980a). Morphological transformation provides a selection for cells that receive BPV DNA.

Cell lines that contain BPV DNA are unusual in that they do not integrate BPV DNA into the cellular chromosomes, but instead carry BPV DNA in an episomal (extrachromosomal) state. Moreover, transformed mouse cells contain 20–100 copies of episomal BPV DNA (Law et al., 1981), providing a natural amplification of the viral vector. Lusky and Botchan (1984) have shown that maintenance of the extrachromosomal state of BPV DNA requires two noncontiguous regions in the BPV DNA as well as an undefined BPV-encoded gene product. When these elements are built into a recombinant DNA molecule, the BPV-recombinant plasmid copy number, once established in a given cell line, is maintained in a stable manner even in the absence of selective pressure.

Sarver et al. (1981) demonstrated that BPV could function as an expression vector by ligating the rat preproinsulin gene to the transforming 69% subgenomic fragment of BPV and establishing cell lines from transformed foci. The cell lines contained the recombinant DNA in an extrachromosomal state at 60–80 copies per cell and secreted proinsulin into the culture medium. Since then, BPV vectors have been used to establish mouse cell lines that produce human β interferon (Zinn et al., 1982; Mitrani-Rosenbaum et al., 1983), hepatitis B surface antigen (Wang et al., 1983), human growth

host-cell DNA in the exact sequence order present in the original cloned DNA, as expected for a normal retrovirus infection.

Vectors can be constructed in which essentially only the LTRs and immediate flanking sequences of the retroviral genome are used. Perkins et al. (1983) used the 5′ and 3′ LTRs of Moloney murine leukemia virus to flank the *Ecogpt* gene, transfected mouse cells, and selected for cells that expressed *gpt* activity. These cells were then infected with helper Moloney murine leukemia virus to produce a recombinant virus stock. When mouse cells were subsequently infected with the resulting virus, 50% of the cells expressed the *gpt* gene. Miller et al. (1983) produced a virus stock that expressed the human HPRT (hypoxanthine phosphoribosyltransferase) gene in a similar fashion. Thus, all the necessary sequences for expression are present in the LTRs; since the HPRT vector can package 6–7 kilobase pairs of extra DNA, it can be used to carry any nonselectable gene up to 7 kb, with the HPRT gene providing a selectable marker. Gilboa et al. (1982) have shown that two genes, *tk* and neo^R, could both be expressed in a Moloney murine leukemia virus vector.

A shortcoming of the preceding experiments is that helper virus was required for the production of recombinant virions. Thus, the viral stock will have a mixture of the two viruses, with the virus titer often composed of approximately 90% helper virus (Miller et al., 1983). In order to circumvent this problem, Mann et al. (1983) have created a mouse cell line that allows the production of recombinant viral stocks free from helper virus. A deletion mutant of Moloney murine leukemia virus was created that lacks the sequence necessary for its RNA to be packaged into a virion (the ψ sequence) but has all the sequences needed to provide, in trans, all other necessary retroviral functions so that defective viruses can be fully complemented by this mutant. They then established a cell line, called $\psi 2$, that contains the mutant viral DNA as part of its genome. Using retroviral DNA containing the *Ecogpt* gene, they demonstrated that these cell lines can act as hosts to package and secrete the recombinant retroviral DNAs as pure retrovirus stocks of approximately 10^4 colony-forming units per ml.

Cepko et al. (1984) have constructed a set of retrovirus vectors known as pZIP-neoSV (shown in Figure 2–4). These vectors are composed of the Moloney murine leukemia virus 5′ and 3′ LTRs (which contain the transcriptional promoter as well as polyadenylation and integration signals), the packaging site, the primer site for reverse transcription, and the 5′ and 3′ splice sites for messenger splicing. The vectors have the neo^R gene to confer G418-resistance in mammalian cells and kanamycin resistance when the vector is propagated as a plasmid in *E. coli,* as well as the SV40 and bacterial (plasmid pBR322) origins of replication. Two different clones were constructed that differ in the position of the cDNA insert. If the cDNA is inserted in the upstream position (as shown in Figure 2–4), it is expressed on an unspliced mRNA while the downstream neo^R gene is expressed on a

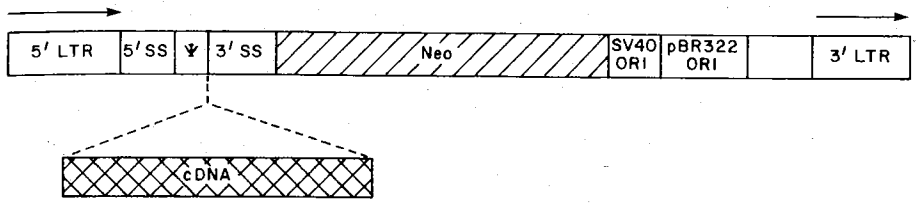

FIGURE 2–4 Map of the retrovirus vector pZIP-neoSV(X)I (redrawn from Cepko *et al.,* 1984). Terminology: LTR, long terminal repeat; 5′ SS, 5′ splice site or splice donor; ψ, virion packaging site; 3′ SS, 3′ splice site or splice acceptor; *neo,* bacterial gene encoding G418 resistance; ORI, origin of replication. The cDNA of a gene to be expressed, in this case, is inserted so that it will be expressed on the unspliced RNA.

spliced transcript. The position of the cDNA insert and neo^R gene can be alternated without effect.

Viral particles are produced by transfecting this recombinant DNA into ψ2 cells. The ψ2 culture fluid is then used to infect 3T3 cells and after selection for G418-resistance, a viral titer of $1–5 \times 10^3$ colony-forming units per ml is reported. Permanent recombinant virus producer lines can be generated by directly selecting for G418-resistance in ψ2 cells. Permanent cell lines yield 10^4 to over 10^6 colony-forming units per ml of culture fluid, making them ideal for long-term recombinant virus production. Spliced and unspliced messages are produced in equal amounts. However, at times, aberrant splicing into the inserted gene can be seen.

It is possible to rescue these vectors from the host cells by a number of means (Cepko et al., 1984). In one protocol, the pZIP-neoSV vectors are transfected into cells that do not allow virus packaging. These cells are then fused with COS cells, a monkey cell line that expresses the SV40 large T antigen. The T antigen induces replication at the SV40 origin of replication and the retroviral vector DNA is generated as episomes, free from the host chromosomes, although sometimes it may carry extra host DNA from the site at which it was integrated.

Another useful property of these and other retrovirus vectors is that genomic DNA-containing introns can be inserted into the vector DNA and the retrovirus finally produced will contain cDNA copies of this genomic clone (Shimotohno and Temin, 1982; Sorge and Hughes, 1982). This occurs because the introns in the genomic DNA are spliced out and the resulting RNA molecules are packaged into virions.

It should be noted that when genes are inserted into retroviral vectors, the genes must not carry their own polyadenylation site. If they did, premature polyA addition would occur and full-length genomic RNA would not be produced (Shimotohno and Temin, 1981). Therefore, polyA addition

sequences must be identified and removed from a gene before it can be inserted into a retroviral vector.

Recently, the pZIP-neo vectors of Cepko et al. (1984) have been used to express the human gene for adenine deaminase (Friedman, 1985). Mouse ψ2 cells, transformed to G418-resistance, expressed 20-fold more human adenine deaminase than endogenous mouse adenine deaminase.

Retroviral vectors that carry two genes with different promoters have now been constructed. This is desirable if one wishes to employ a specific inducible promoter to produce a protein. However, an unexpected problem arose when Emerman and Temin (1984) constructed vectors with the *neo*^{R} gene transcribed from the LTR and the HSV *tk* gene transcribed from its own promoter. Surprisingly, they found that if only one copy of the vector was present in the host cells, either one of the two genes, but not both, was expressed. This effect is epigenetic and reversible, as rescued viruses can change which gene is expressed after reinfection. This phenomenon is not understood and may be cell-specific, as other laboratories have reported different findings (Miller et al., 1984).

The rat growth hormone with its own inducible promoter was inserted into a murine retrovirus vector downstream of the selectable marker HPRT, which was transcribed from the 5′ LTR (Miller et al., 1984). This vector was transfected into HPRT$^-$ cells, and the HPRT$^+$ colonies were selected in HAT medium. The HPRT$^+$ cells were then infected with helper virus and high titers (up to 10^6 per ml) of recombinant virus were obtained and used to infect HPRT$^-$ cells. In the cells infected by the recombinant virus, the growth hormone gene was inducible by both glucocorticoids and thyroid hormone. The growth hormone gene could be expressed and induced when placed in either orientation. The recombinant DNA was found in a single copy per host cell, and in this case, both the growth hormone and HPRT genes were expressed.

The ψ2 cell line, a mouse cell line, produces virions with a host range limited to rodents. Sorge et al. (1984), Cone and Mulligan (1984), and Miller et al. (1985) have now produced helper-free cell lines that package viruses of very wide host range. Recombinant viruses can now be produced that are able to infect avian, rodent, canine, simian, and human cells.

In conclusion, retroviruses offer a number of significant advantages as expression vectors. Once virus is produced, the introduction of the recombinant DNA into cells is very efficient, and the integration into the genome is precise, lacking the rearrangements sometimes seen after transfection of DNA into cells. Also, genes introduced into cells in retroviral vectors as viruses appear to be more efficiently expressed than the same genes transfected as naked DNA (Hwang and Gilboa, 1984). Once integrated, the DNA appears to be very stably maintained. One disadvantage is that the retroviral DNA usually inserts in only one or a few copies. However, if a high gene copy number is desired in order to increase gene expression, the copy number can be increased after infection by use of a retroviral vector